PRODUCTION OF ABE (ACETONE-BUTANOL-ETHANOL) FROM POME BY *Clostridium beijerinckii*

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ABSTRACT

Malaysia was one of the biggest producers and exporters of palm oil and palm oil products (Malaysian Palm Oil Council, 2012). Thus, the total oil palm cover has increased with a corresponding increase in palm oil production. As a result, palm oil waste which was a by-product of the milling process will also increase. Due to the presence of high total solids in palm oil mill effluent (POME), attempts have been made to convert this waste into valuable products such as feed stock (Rupani *et al.*, 2010). Consequently, purpose of this research was to study about the production of acetone-butanol-ethanol (ABE) solvents using POME by *Clostridia beijerinckii*. These ABE solvents which are consist of acetone, butanol and ethanol have each one benefits such as butanol used for biofuel, acetone used for making plastic and ethanol can be used as disinfectant. The culture was grown in RCA and then 10% v/v of the culture was transferred to RCM for preparation of inoculum medium. After that, 10% v/v of inoculum medium was transferred into fermentation medium which consists of 90% of POME. In this research, POME would be used as substrate medium. Then, the fermentation medium was run to find the optimum pH for fermentation medium (initial pH 5.4 until 6.2), concentration of POME (80% to 100% v/v) and temperature of fermentation (27°C to 47°C). The highest value of total ABE obtained was 0.771 g/L with 90% concentration at condition of pH 5.8 of 37°C.

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LIST OF ABBREVIATIONS

ABE	Acetone-Butanol-Ethanol
CPO	Crude Palm Oil
DNS	Dinitrosalicylic Acid
FID	Flame Ionization Detector
GC	Gas Chromatography
min	Minute
OD	Optical Density
POME	Palm Oil Mill Effluent
RCA	Reinforced Clostridia Agar
RCM	Reinforced Clostridia Medium
UV-VW.	Ultraviolet-Vis Spectroscopy

1 INTRODUCTION

1.1 Background of research

The revival of A–B–E fermentation was currently being inspired by the consideration of butanol for biofuel. (Lee *et al*, 2008a). During early 20th century through World Wars I and II, ABE fermentation was significant for the production of butanol and acetone solvents. However, its use has declined since the 1950s due to increasing costs of the substrate molasses and unable to compete economically with petrochemically produced ABE.

As the result, the following factors which severely affect the economics of ABE fermentation were identified which high cost of substrate, low product concentration (<20 g/L), low reactor productivities (<0.3 g/L/h), low ABE yields (0.28–0.33); and an escalated cost of butanol recovery by distillation which was the only technique for recovery at that time. Additional factors such as bioreactor costs, interest rate on the borrowed capital, and rate of return on the investment were also identified as factors which affect the price of fuels derived from renewable resource (Qureshi & Blaschek, 2001). Nevertheless, in 1973, because of oil price increases, this crisis led to renewed interest in solvent production by ABE fermentation (Tashiro & Sonomoto, 2010).

Recently, there has been increased interest on using renewable resources as starting materials. Biomass is a widely available substrate which considered an environmentally friendly process (Lo´pez-Contreras, 2001). Kalil *et al*. (2003) mentions that palm oil is one of the world's leader edible oils produced by South East Asian and African countries. Palm oil was used for producing various products of food, pharmaceutical and oleo-chemicals. Its production generates various wastes and one of it was palm oil mill effluent (POME). POME was selected as substrate for ABE fermentation because of cheap raw material and to solve environmental problem. Furthermore, its mixture contents were suitable for growth of *C. beijerinckii*.

1.2 Motivation and problem statement

As the natural resources such as oil decreasing over the time; the researchers have been looking for other alternatives by producing biofuel from renewable sources that can be updated through fermentation. The acetone-butanol-ethanol (ABE) fermentation has attracted the attention of the researchers because it has the potential to produce chemicals and liquid fuels. Due to the cost of materials for medium preparation which was most expensive, ABE fermentation could not survived. The other solution was to find another substrate (fermentation medium) that can carry on the fermentation process.

Malaysia is the world largest Palm Oil Producing Country. Approximately 99.85 MT/yr of Palm Oil Mill were produced in 2011. Because of POME gave pollution of water ways, it had an impact on the environment. Thus, the industry faced a major problem, as it virtually lacked any proven technology to treat POME (MPOB, 2012).

Therefore, some ways to solve this problem was found. Because of sedimented POME with reduction of water content contains higher concentrations of lignocelluloses and other insoluble materials; it could support growth of *Clostridia beijerinckii* for ABE fermentation (Kalil *et al*., 2003). Palm oil mill effluent (POME) also seems has a great potential as a substrate for ABE fermentation because it contains a mixture of carbohydrates including starch, hemicelluloses, sucrose and other carbohydrates that can be utilized by saccharolytic *clostridia*.

Other than that, POME was a sustainable resources and it was a cheap raw material that can be easily obtained. (Kalil, 2003). Lorestani (2006) estimated that in Malaysia about 53 million m^3 POME was being produced every year based on palm oil production in 2005 (14.8 million tonnes). While Yacob *et al.* (2005) estimated that about 0.5-0.75 tonnes of POME will be discharged from mill for every tonne of fresh fruit bunch. If there is no strategic mechanism on reusing the waste, it could further create other problems which would harm the environment.

1.3 Objectives

The following are the objectives of this research:

- To study the effect of substrate concentration, temperature and initial pH of substance on solvent production by *Clostridium beijerinckii*.

1.4 Scope of research

To achieve objective of this study, there were several scope that have been identified:

i) Effect of substrate concentration on solvent production: 80%, 85%, 90%, 95% and 100%

ii) Effect of temperature on solvent production: 27°C, 32°C, 37°C, 42°C and 47°C

iii) Effect of initial pH on solvent production: 5.4, 5.6, 5.8, 6.0 and 6.2

iv) Glucose consumption by *Clostridium beijerinckii* along the fermentation process.

1.5 Rationale and significance

1. This research focused on the production of solvents which are ABE (acetone-butanol-ethanol) through fermentation process. In this research, POME was used as the substrate fermentation for ABE production.

2. The revival of ABE fermentation was presently being inspired by the consideration of butanol for biofuel. Butanol has been proposed as a gasoline additive or even as a complete gasoline replacement. (Li *et al.*, 2011). Butanol can also be used as a blended additive to diesel fuel to reduce soot emissions.

3. Acetone was a first-class solvent for most plastics and synthetic fibers including of polystyrene, polycarbonate and some types of polypropylene. For ethanol, it was used as fuel same as butanol. It also used as a solvent for various organic compounds and as disinfectant.

2 LITERATURE REVIEW

2.1 A-B-E (Acetone-Butanol-Ethanol)

According to Lee *et. al* (2008), biological production of acetone-butanol-ethanol was one of the largest industrial fermentation processes early in the 20th century. In 1861 butanol production through microbial fermentation was reported for the first time by Pasteur. This was followed by Schardinger in 1905 reporting production of acetone by fermentation. From 1912 to 1914 strains of Clostridium acetobutylicum were isolated by Chaim Weizmann which had the ability of fermenting starchy substrate. These cultures produced higher butanol yields than the cultures of Fernbach.

During World War I and II the ABE fermentation industry had the largest growth in Europe and USA. Acetone was used for manufacturing cordite, a smokeless powder used in ammunition. Butanol was also used as a solvent for quick drying lacquers used in the automobile industry for painting cars. 2/3 of the overall butanol and 1/10 of acetone was produced by fermentation in USA at the end of World War II in 1945. At this time, large-scale production of acetone and butanol through ABE fermentation was implemented in the former Soviet Union. Maize, wheat and rye were used as major substrates. Large-scale fermentation processes were also operated in countries such as China, Japan, Australia and South Africa. (Lee *et. al*, 2008)

After World War II the petrochemical industry flourished at an unprecedented rate and so a huge decline in the ABE fermentation industry was observed. Through petrochemical industry large quantities of much cheaper acetone und butanol were available on the market. This fact resulted in an uneconomical ABE fermentation process. Most of the fermentation industry in western countries ceased to exist by 1960. In South Africa, Russia and China the ABE fermentation was carried out until 1980 to 1990 because of cheap supply of molasses as substrate and a relatively small availability of acetone and butanol from petrochemical industry (Lee *et. al*, 2008).

Nowadays, the interest in this fermentation process has come back due to depleting oil reserves and high oil price. The main aims in today's research are to improve the complete process by using genetically modified strains and cheaper renewable substrates. Some other important renewals must be the research into better cultivation and efficient product removal techniques.

2.1.1Industrial used of ABE fermentation

Acetone with formula OC $(CH_3)_2$ was the organic compound. Acetone is a colorless, mobile and flammable liquid of the ketones. Acetone was miscible with water and virtually all organic solvents, it serves as an important solvent in its own right, typically the solvent of choice for cleaning purposes in the laboratory. Acetone was also used as solvents and in the production of the rubber monomers, butadiene and dimethyl butadiene (Ezeji *et al*., 2003). In industrial solvent, acetone was found in the paint, lacquer & varnish industry, rubber industry, plastics industry, dyeing industry, celluloid industry, photographic & explosives industry & in the manufacture of artificial silk & synthetic leather.

Butanol is a chemical which has excellent fuel characteristics. It has a higher calorific value than ethanol, and a low freezing point (Qureshi & Blaschek, 2000). Butanol has recently been proposed as a gasoline additive, or even as a complete gasoline replacement (Lee *et al.*, 2008a). Butanol was the most promising solvent compared to acetone and ethanol due to its higher price, better fuel extender than ethanol, low vapor pressure; low miscibility with water and it was completely miscible with diesel fuel even at low temperatures (Qureshi, 2001).

Butanol or Bio-butanol has been recognized as a potential fuel from renewable resources. Bio-butanol was formed via ABE fermentation from renewable feedstocks using *Clostridium beijerinckii* in anaerobic conditions. Butanol gives several benefits compared to ethanol as a bio-fuel such as higher energy content, lower vapor pressure, and lower hygroscopy (Kraemer, 2010). Butanol was also used as a solvent for quick drying lacquers used in the automobile industry for painting cars. Butanol with acetone was used in the making of explosive materials. N-butanol was used in the manufacture of plasticizers, brake fluids, urea-formaldehyde, extractants and petrol additives (Priya, 2009).

Ethanol is a clear, colorless, very mobile liquid, a clean-burning and high-octane motor fuel that is produced from renewable sources. Other than that, it was used as a solvent, extractant, and antifreeze. At its most basic, ethanol is grain alcohol, produced from crops such as corn. Because it is domestically produced, ethanol helps reduce America's dependence upon foreign sources of energy. Unblended 100% ethanol is not used as a motor fuel; instead, a percentage of ethanol is combined with unleaded gasoline (American Coalition for Ethanol).

2.2 Palm oil

After being one of the biggest producers and exporters of palm oil and palm oil products, Malaysia has an important role to play in fulfilling the growing global need for oils and fats sustainably. Malaysia currently accounts for 39 % of world palm oil production and 44% of world exports. Today, 4.49 million hectares of land in Malaysia was under oil palm cultivation; producing 17.73 million tonnes of palm oil and 2.13 tonnes of palm kernel oil (“MPOC”, 2012).

Oil palm bears both male and female flowers on the same tree as it was a monoecious crop. Each tree produces 1000 to 3000 fruitlets per bunch weighing between 10 and 25 kilograms. Each fruitlet was almost spherical or elongated in shape. Generally, the fruitlet was dark purple, almost black and the colour turns to orange red when ripe. Each fruitlet consists of a hard kernel (seed) enclosed in a shell (endocarp) which was surrounded by a fleshy mesocarp ("MPOC", 2012).

An oil palm plantation was capable of assimilating up to 36.5 t of dry matter per hectare per year, which was higher than the 25.7 t assimilated by natural rainforest. The oil palm was thus more effective than the rain forest in generating new biomass, which has wide uses for wood-replacement and was also a potential source of renewable biofuel. ("MPOC", 2012).

Figure 2–1: Illustration Oil palm tree

2.2.1 Palm oil mill effluent (POME)

Characteristics of palm oil mill effluent depend on the quality of the raw material and palm oil production processes in palm oil mills. There has categorized three major processing operations responsible for producing the POME. Sterilization of FFB, clarification of the extracted CPO, and hydrocyclone separation of cracked mixture of kernel and shell hydrocyclone contributes about 36, 60 and 4% of POME respectively in the mills (Rupani *et. al*, 2010).

According to Hii *et al* (2012), palm oil mill effluent (POME) was rich in carbohydrates, proteins, nitrogenous compounds, lipids, minerals, cellulose, hemicelluloses and lignin. It can be used naturally as a fermentation medium, either for cellulase or other value-added product fermentation. Ma (2000), stated that because of the organic acids produced in the fermentation process, palm oil mill effluent was low in pH about 4-5. It also contains large amounts of total solids (40,500 mg/ l), oil and grease (4000 mg/ l).

POME can be sustainably reused as a fermentation substrate in the production of various metabolites, fertilizers and animal feeds through biotechnological advances (Wu, 2009). Hii (2012) said that several researchers was determined the proximate composition of POME found that POME was very rich in carbohydrates, proteins, nitrogenous compounds, lipids and minerals. Kalil (2003) also agreed and said that sedimented POME with reduction of water content contains higher concentrations of lignocelluloses and other insoluble materials which supported growth of *Clostridia*. Moreover, sedimentation of POME assisted to eliminate traces of oil and soluble toxic substances leaving less inhibitory POME. (Kalil, 2003)

Table 2-1: Characteristic and composition of POME

Components	Range/average*
pH	3.5-4.7
Oil and grease	4000
Biochemical oxygen demand (BOD_5)	10 250-43 750
Chemical oxygen demand (COD)	16 000-100 000
Total solids	11 450-164 950
Suspended solids	410-60 360
Volatile solids	8670-154 720
Total volatile solids	34 000
Ammoniacal nitrogen	35
Total nitrogen	200-500
Lignin	2900-7890
Cellulose	250-8000
Reducing sugar	4230-6720

*All units in mg/L except pH (Source: Hii, 2012)

2.3 *Fermentation*

2.3.1 *Clostridium beijerinckii*

Clostridia are rod-shaped, spore-forming Gram positive bacteria and typically strict anaerobes. Solventogenic clostridia can utilize a large variety of substrates from monosaccharides including many pentoses and hexoses to polysaccharides (Jones and Woods, 1986). In *Clostridium beijerinckii* (and probably also other butanologenic strains), the solventogenic genes are located on the chromosome (Zverlov *et al*., 2006)

Hiu *et al.* (1987) mentions that strains of *Clostridium beijerinckii* which formerly known as *Clostridium butylicum* can produce isopropanol in addition to acetone, butanol and ethanol. *C. beijerinckii* was the gram-positive, spore forming, mesophilic, motile, rod-shaped bacteria with oval, sub-terminal spores and anaerobic clostridia constitute a diverse group of species (Shi & Blaschek, 2008).

2.3.2 *Anaerobic Fermentation*

Anaerobic was composting without oxygen which results in fermentation. "Anaerobic composting" describes the process of putrefactive (cause odor nuisance) breakdown of organic matter by reduction in the absence of oxygen where end products such as CH_4 and hydrogen sulfide (H_2S) are released. This condition causes organic compounds to break down by the action of living anaerobic organisms. Anaerobic composting may be accomplished in large and well composting systems. These should contain 40% to 75% moisture, into which little oxygen can penetrate, or 80% to 99% moisture so that the organic material was a suspension in the liquid. When materials are composted anaerobically, the odor nuisance may be quite severe. ("Compost fundamentals")

2.3.3 *Batch Fermentation*

It takes 2–6 days to complete batch fermentation depending on the condition and the type of substrate employed. The final total concentration of solvents produced ranges from 12 to 20 g/L in batch fermentation, which can be separated from the fermentation broth by distillation (Lee *et. al*, 2008).

2.3.4 ABE Fermentation

ABE hetero-fermentation produces acetate, butyrate, ethanol, and acetone, as well as butanol. The metabolism of ABE producing clostridia can be divided into the following 2 distinct phases: acidogenesis (acid-production) and solventogenesis (solvent-production) during the exponential and stationary phases of growth. The metabolic pathways of ABE-producing clostridia are summarized in **Figure 2.2**.

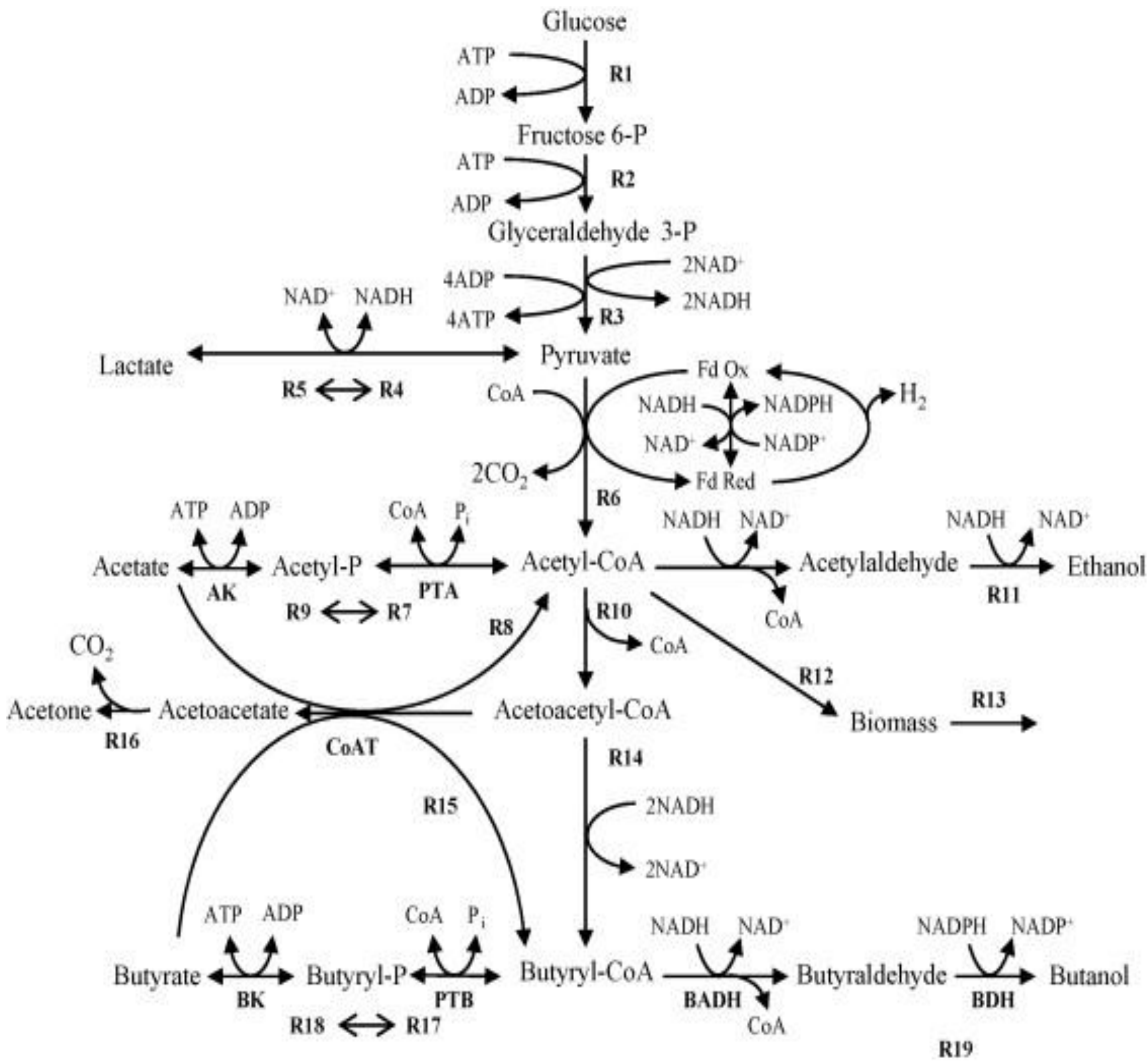

Figure 2-2: Metabolic pathway of acetone-butanol-ethanol fermentation

(Source: Run *et. al*, 1988)

C. beijerinckii produces hydrogen, carbon dioxide, acetate and butyrate through a first growth phase (acidogenic phase) in carbohydrate batch culture. During acidogenesis, acetate and butyrate are produced from acetyl-CoA and butyryl-CoA, respectively; ATP was also produced. The four primary enzymes involved in the formation of butyryl Co-A are thiolase, B-hydroxy butyryl Co-A dehydrogenase, crotonase and butyryl Co-A dehyrogenase.

Accumulation of these organic acids reduces the culture pH. Medkor (2010) mentions that a decrease in the pH of the culture medium was occurred on acidogenic phase. Decrease in pH was needed to start solventogenesis. Enough acids have to be formed before pH decrease or otherwise the solventogenesis will be unproductive ("REBEL WP7", 2009). The organic acids are then re-utilized insolventogenesis, when the culture pH begins to rise. The re-utilization of acetate and butyrate was generally considered to occur via the acetoacetyl-CoA: acetate/butyrate: CoA transferase (CoAT) pathway and the reverse pathway generates the organic acids.

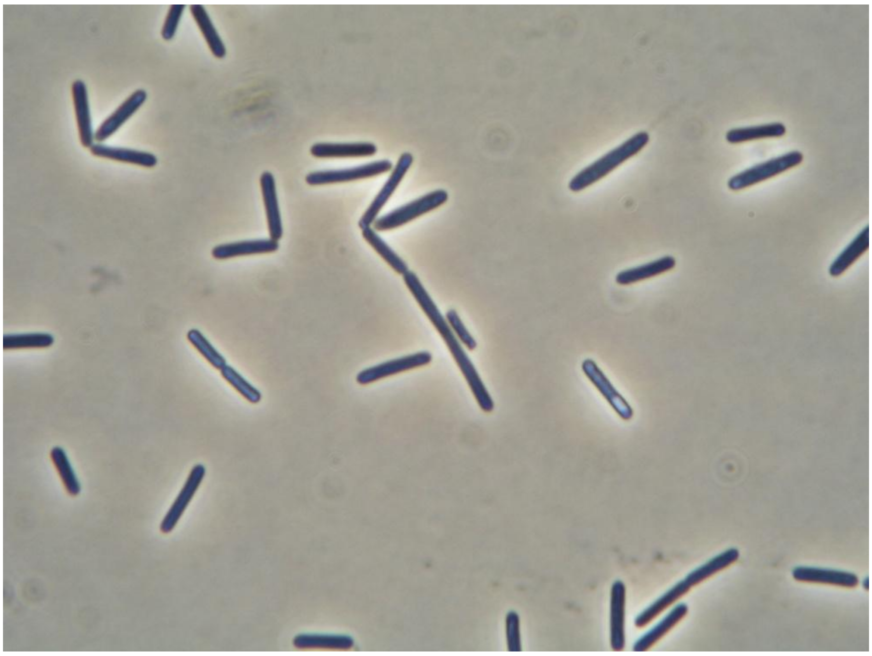

Figure 2-3: Growth phase (acid production)

(Source: Melzoch *et. al*, 2010)

During solvent production, acetyl Co-A and butyryl Co-A become main intermediates for ethanol and butanol production. These pathways produce acetaldehyde and butraldehyde, respectively as intermediates. The reduction of butryl Co-A to butanol was mediated by butraldehyde dehydrogenases and butanol dehydrogenase. Acetoacetyl Co-A transferases convert the acetoacetyl Co-A to acetoacetate.

Acetone, n-butanol, ethanol, and isopropanol (solvents) are characteristic products of several Clostridium species (Run *et. al*, 1988). Recently, ABE producing clostridia were reclassified into 4 species which are *Clostridium acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* by using the molecular biological methods of 16S rRNA sequencing, DNA fingerprinting, and DNA-DNA hybridization (Tashiro and Sanomoto, 2010).

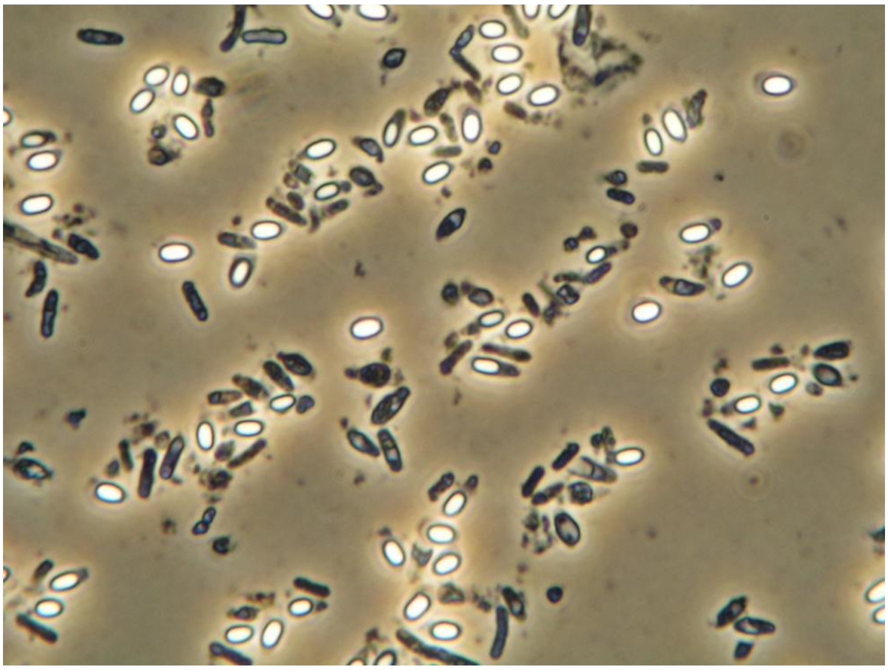

Figure 2-4: Sporulation (solvent production)

(Source: Melzoch *et. al*, 2010)

2.4 Effect of Fermentation Parameters

2.4.1 pH

According to Kim *et al*. (1984), the pH of the medium was very important to the biphasic acetone–butanol fermentation. In acidogenesis, rapid formation of acetic and butyric acids causes a decrease in pH. Solventogenesis starts when pH reaches a critical point, beyond which acids are reassimilated and butanol and acetone are produced. Therefore, low pH is a prerequisite for solvent production. However, if the pH decreases below 4.5 before enough acids were formed, solventogenesis would be brief and unproductive. Ahmed *et al.* (1988) conclude that *C. beijerinckii* produces butanol at more rapid growth rates in acid medium and at slower growth rates in neutral medium. Ethanol was produced at all growth rates and at both pH levels.

POME cultures that work at lower pH compared to RCM culture produced more solvent and this agreed with the findings of Girbal and Soucaille (1998) who found that culture with high pH produced more acids than solvent itself. According to Kalil *et al*. (2003), clostridial strains other than *C. acetobutylicum* showed that production of ABE was optimum at the initial pH values of 5.0 to 6.2. The initial pH of POME concentration was increased to pH 5.8 before used for ABE fermentation medium in order to get high yield of solvent.

2.4.2 Temperature

According to Al-Shorgani *et al.* (2012), the yield of ABE was higher when POME treated by autoclaving was used. This might be due to the effect of high temperature, resulting in hemicellulosic degradation and lignin transformation. It shows that, the effect of temperature was important to produce high ABE solvent. Other than that, Boonsombuti *et al.* (2013) mentioned the incubation temperature was maintained at 37°C. Lopez-Contreras *et al.* (2001) also agreed that the optimal temperature for incubation of the cultures was suitable at 37°C.

2.4.3 POME concentration

Kalil *et al*. (2003) agreed that sedimented POME at 90% concentration was the optimum concentration for ABE production by fermenting *Clostridia*. According to previous result, 90% POME concentration (sedimented) seen produced the highest ABE compared to POME 70% and POME 80%. Sedimentation of POME assisted to eliminate traces of oil and soluble toxic substances leaving less inhibitory POME. Sedimented POME was suitable to be used because of high lignocellulose concentrations and other insoluble materials needed for production of ABE solvent.

Figure 2-5: Palm oil mill effluent

3 MATERIAL AND METHODS

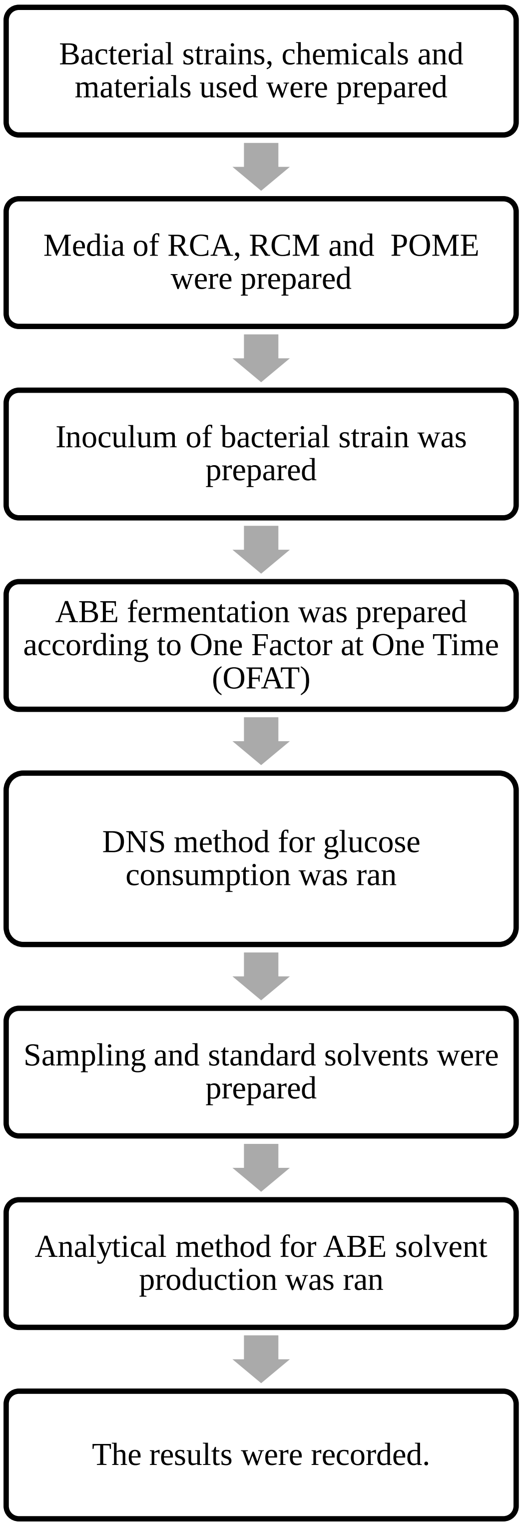

Figure 3-1: Flow chart of methodology

3.1 Equipment

3.1.1 Autoclave

Autoclave model Hirayama HVE-50 was used to sterilize equipment by subjecting them to high pressure saturated steam at 121°C for 15 to 20 minutes. Mode that common being used for autoclave was 2 which was for culture medium. Before use the equipment, water level of that equipment must be checked first and used an appropriate water type whether de-ionized water or distilled water depend on what type of autoclave used. In this research, this equipment was the main equipment for sterilization.

3.1.2 Biohazard Safety Cabinet

Biohazard safety cabinet brand ESCO, model AC2-4EI was used as the place where the experiment was conducted. This Biohazard safety cabinet provide ideal partical-free, bacteria free, clean air environment that needed for laboratory work testing. Other than that, it also does sterilization of various microorganisms in the interior of laminar flow. UV lamp was also used as a germicidal lamp.

3.1.3 Incubator shaker

The incubator shaker used was brand Infors Multitron 11. This incubator was used to incubate the Reinforced Clostridial Medium and POME for ABE production. The optimum incubation temperature used was at 37 °C with a 150 rpm orbital shaking rate without pH control for RCM (Boonsombuti *et al*., 2013).